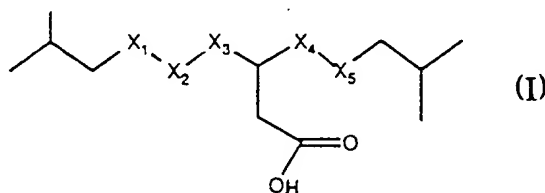




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(57) Abstract

Non-peptidic Leu-Asp-Val (LDV) surrogates of formula (I), wherein  $X_1$  to  $X_5$  are the same or different C, N, O or S atoms, at least two of them being C, and the  $X_1$ - $X_5$  chain may be optionally substituted by radicals selected from halogen, hydrocarbyl, oxo, thioxo, amino and carboxyl, or the  $X_1$ - $X_5$  chain or part thereof may form part of a heterocyclic ring, and pharmaceutically acceptable salts thereof, inhibit cellular or molecular interactions which depend on recognition of the LDV sequence, and are useful for the treatment of inflammation and disorders involving interference with cell-matrix or

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# NON-PEPTIDIC SURROGATES OF THE LDV SEQUENCE AND THEIR USE IN THE TREATMENT OF INFLAMMATION, AUTOIMMUNE DISEASES AND TUMOUR PROGRESSION

## Field and Background of the Invention

The present invention relates to novel Leu-Asp-Val (LDV) surrogates, to their preparation and to pharmaceutical compositions comprising them for treatment of several disorders.

The ability of blood cells to adhere to and to interact with other cells or with components of the extracellular matrix (ECM) is fundamental for maintaining cell function and tissue integrity through signals delivered between and within the communicating cells. Cell-cell and cell-ECM adhesive interactions are regulated primarily by a super family of cell adhesion receptors, the integrins, which are widespread on most cell types including lymphocytes, tumor cells and platelets.

The integrins are heterodimeric molecules consisting of an alpha ( $\alpha$ ) and a beta ( $\beta$ ) subunits which are non-covalently linked. Eleven  $\alpha$  and six  $\beta$  subunits have been identified. The pairing of  $\alpha$  and  $\beta$  subunits may be cell-type specific and determines ligand specificity.

The integrins play an important role in linking the ECM with the intercellular actin-containing cytoskeleton: the extracellular portion mediates the binding of adhesive proteins, and the intracellular portion interacts with elements of the cytoskeleton.

The exit of cells from blood vessels and their ensuing tissue localization depends on receptor-mediated recognition of endothelial cells, their basement membranes and the ECM. The ability of various cell types to recognize and attach to components of the ECM may therefore be considered as an essential physiological feature of homeostasis.

Fibronectin (FN), a well characterized ECM-derived cell-adhesive glycoprotein, is present in a variety of

matrices and is synthesized and secreted by a variety of cell types as a 230-250 kDa cross-linked dimer composed of three types of internal repeats: I, II, and III. FN is involved in processes that include wound healing, embryonic cell migration and differentiation, cell activation, proliferation, and adhesion. Cell binding to immobilized FN is mediated primarily by surface integrins of the  $\beta_1$  (CD29; very late antigens (VLA))-subfamily of receptors, including VLA-3, -4 and -5. The interaction of FN with these integrins relies on its central cell-binding domain and depends on the cell adhesion motif Arg-Gly-Asp (RGD), which is recognized primarily by the  $\alpha_5\beta_1$  (VLA-5) integrin. Nevertheless, additional adhesion epitopes on the alternative spliced sequences of the CS1 region of the type III connecting segment (IIICS) of FN were recently characterized. The minimal tripeptide essential for the cell-adhesive functionality of this domain is the Leu-Asp-Val sequence (LDV; Scheme 1 herein; Humphries et al, 1990), the binding of which is primarily mediated by the  $\alpha_4\beta_1$  integrin (VLA-4; Nojima et al, 1990). This domain was found to be recognized by integrin receptors of various cell types, such as those on tumor cells and lymphocytes (Guan et al, 1990; Shimizu et al, 1990; Postigo et al, 1991; Roldan et al, 1992). Interestingly, the VLA-4 integrin was also implicated in the recognition of vascular cell adhesion molecule-1 (VCAM-1) on endothelial cells, although this binding site was distinct from that recognized on FN (Elices et al, 1990).

Inhibitors of VLA-4 recognition of FN may thus interfere with cell migration from the circulation to inflammatory sites. Indeed specific interference with VLA-4 activity, was found to inhibit experimental autoimmune encephalomyelitis as well as the elicitation of immune-cell mediated contact sensitivity reaction in mice.

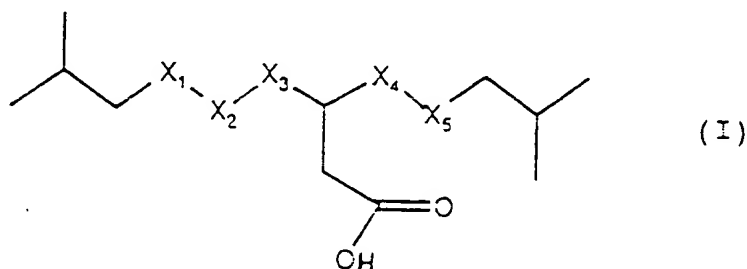
Recently we have designed and produced several structural nonpeptidic mimetics of the RGD-sequence made of a spacer chain, linking a guanidinium and carboxylic acid groups (International PCT Application PCT/US92/09951). These compounds (e.g. 5-N-(6-guanidinohexanamide)-pentanoic acid)

exhibit considerable affinity for the RGD-dependent platelet integrin  $\alpha_{IIb}\beta_3$  inhibit platelet aggregation, and interfere with RGD-dependent adhesion of CD4<sup>+</sup>T lymphocytes and tumor cells to immobilized FN and vitronectin. In vivo, the RGD surrogate effectively inhibited the elicitation of a CD4<sup>+</sup>T cell-mediated inflammatory reaction in mice, indicating that the proteolytically stable RGD-mimetics may serve as useful therapeutic agents in a variety of pathologic processes which depend on RGD recognition .

### Summary of the Invention

It has now been found according to the present invention that certain LDV analogues are effective inhibitors of cellular or molecular interactions which depend on LDV-sequence recognition. These LDV analogues are herein referred to as "LDV surrogates".

The present invention thus relates to compounds of the general formula I



wherein

$X_1$  to  $X_5$  are the same or different C, N, O or S atoms, at least two of them being C, and the  $X_1$ - $X_5$  chain may be optionally substituted by radicals selected from halogen, hydrocarbyl, oxo, thioxo, amino and carboxyl, or the  $X_1$ - $X_5$  chain or part thereof may form part of a heterocyclic ring, and pharmaceutically acceptable salts thereof.

In a preferred embodiment,  $X_1$ ,  $X_2$  and  $X_4$  represent carbon atoms,  $X_5$  is NH and  $X_3$  is NH or S, and the  $X_1$ - $X_5$  chain is substituted by one or two oxo groups. In other preferred embodiment,  $X_1$ ,  $X_3$  and  $X_4$  are carbon atoms,  $X_2$  is S and  $X_5$  is NH.

The invention further relates to methods for the preparation of the LDV surrogates of the invention.

The LDV surrogates of the invention have various applications related to their inhibition of biological interactions dependent on LDV-sequence recognition, such as integrin-mediated cell functions. Thus the invention also relates to pharmaceutical compositions comprising the LDV surrogates for the treatment of several disorders, such as inflammatory disorders, and disorders involving interference with cell-matrix or cell-cell dependent immune processes, such as autoimmune diseases, allergy, graft-versus-host and related reactions, and inhibition of metastasis and tumor progression.

#### Brief Description of the Drawings

Fig.1 shows specificity of inhibition of T cell adhesion to fibronectin (FN) and laminin (LN) by the following inhibitory compounds: peptides GRGDSP AND EILDVPST, the LDV surrogates AC-16 and EG-45, and the comparison compounds AC-22 and BL-34.

Fig.2 shows analysis of inhibition of T cell adhesion to FN by the inhibitory compounds as in Fig.1, at concentrations of 800, 400 and 200 µg/ml.

Fig.3 shows dose-dependent inhibition of T cell adhesion to FN by the EILDVPST peptide (white symbols) and the corresponding EG-45 surrogate (black symbols).

#### Detailed Description of the Invention

In designing the LDV surrogates according to the present invention, it was considered that the peptide amino and carboxyl end groups are not involved in recognition by its respective integrin and therefore do not affect its inhibitory activity, as is the case for RGD. It was also further considered that the major contribution of the LDV-containing peptides for binding to their putative integrin-recognition sites on integrins depends on the interaction of the carboxylate moiety of Asp (aspartic acid) inside an hydrophobic pocket in which the side chains of Leu are Val

are bound. The preferred conformation of this tripeptide is most probably dominated by the S-trans configuration of the two peptide bonds, and therefore the compounds prepared according to the invention have preferably two hydrophobic side chains and a carboxylate moiety in the same relative orientations they have in the LDV peptide.

As used herein the term "LDV surrogates" refers to novel compounds of the general formula I herein, and pharmaceutically acceptable salts thereof.

The term "hydrocarbyl" as employed herein includes straight or branched  $C_1$ - $C_{12}$  alkyl and  $C_2$ - $C_{12}$  alkenyl or alkynyl radicals, such as methyl, ethyl, propyl, isopropyl, butyl, isobutyl, sec-butyl, pentyl, vinyl, allyl and ethynyl;  $C_3$ - $C_7$  cycloalkyl and cycloalkenyl radicals, such as cyclopropyl, cyclopentyl, cyclohexyl and cyclohexenyl; and  $C_6$ - $C_{14}$  aryl radicals, such as phenyl and naphthyl.

The heterocyclic rings comprising the  $X_1$ - $X_5$  chain or part thereof include saturated or unsaturated rings, optionally containing additional O, S and/or N atoms, and may be optionally substituted by halogen, hydrocarbyl, oxo, thioxo, amino or carboxyl. An example is the compound herein designated NG-93, which contains a 13-membered ring.

In a preferred group of compounds according to the invention one or more of  $X_1$  to  $X_5$  are NH and one or more are C atoms substituted by oxo groups, such as the compound herein designated AC-16 in which  $X_3$  and  $X_5$  are -NH,  $X_1$  is  $>CH_2$  and  $X_2$  and  $X_4$  are carbonyl groups, of the formula depicted in Scheme 1 herein.

In another preferred group of compounds, one or more of  $X_1$  to  $X_5$  are -NH, one is O or S and one or more are C atoms substituted by oxo groups, such as the compound herein designated LB-1, in which  $X_3$  is S,  $X_5$  is -NH,  $X_1$  and  $X_2$  are  $>CH_2$  and  $X_4$  is carbonyl, and the compound herein designated EG-45, in which  $X_2$  is S,  $X_5$  is -NH,  $X_1$  and  $X_3$  are  $>CH_2$  and  $X_4$  is carbonyl (Scheme 1).

In another preferred embodiment, the atoms of the  $X_1$ - $X_5$  chain together with the C atom adjacent to  $X_5$  form

part of a heterocyclic ring, such as the compound herein designated NG-93, in which  $X_3$  and  $X_5$  are  $-NH$ ,  $X_2$  and  $X_4$  are carbonyl and they form part of a 13-membered heterocyclic ring (Scheme 1).

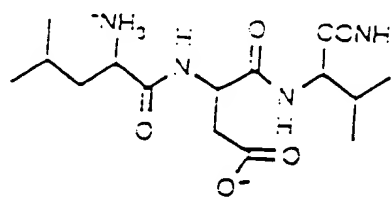
The compounds of the invention may be prepared by several procedures.

LDV analogues wherein  $X_4$  is carbonyl and  $X_3$  is nitrogen, e.g. compound AC-16, are prepared from protected L-Asp, e.g., t-butyloxycarbonyl Asp (O-benzyl) wherein amide bonds (i.e.,  $X_5$  is  $-NH$  and  $X_2$  is carbonyl) are prepared by coupling with carbodiimide as shown in Scheme 2. Ester bonds ( $X_5$  is oxygen) and thioesters are also prepared by carbodiimide coupling. As a control, LEV analogues, such as the compound AC-22, are prepared according to the same synthetic scheme using as the starting material protected L-Glu, e.g. t-butyloxycarbonyl Glu-(O-benzyl).

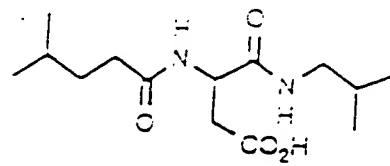
Cyclic peptides, e.g. NG-93, are prepared by stepwise synthesis on an oxime resin starting from Val attached to the resin and coupling of Asp followed by Leu. The peptide is then cleaved from the resin by an excess of t-Boc  $\Omega$ -amino acid. Removal of the t-butyl protecting groups followed by cyclization (DCC, 1-hydroxybenzotriazole) affords the O-benzyl protected cyclic peptide which is purified by HPLC. Hydrogenolysis of the O-benzyl group (Pd/C 10%) at atmospheric pressure of  $H_2$  gives the cyclic compound.

LDV analogues wherein  $X_3$  is sulfur and  $X_5$  is nitrogen, oxygen or sulfur, e.g. compound LB-1, are prepared by a Michael addition of 4-methyl-1-pentanethiol to benzyl maleate. The addition takes place preferentially  $\beta$ - to the ester group. Coupling of the free carboxyl group with isobutyl amine, or alcohol or thiol (dicyclohexylcarbodiimide, 1-hydroxy-benzotriazole) affords the benzyl-protected analogue. The benzyl group is removed by catalytic hydrogenation and the product, e.g. compound LB-1, is purified, e.g. by recrystallisation.

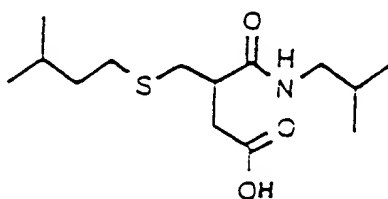




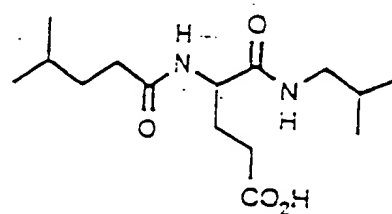
LDV-NH<sub>2</sub> peptide.



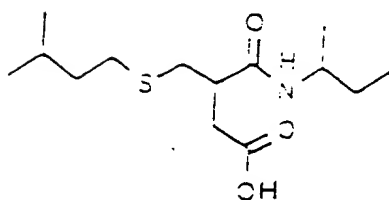
AC-16



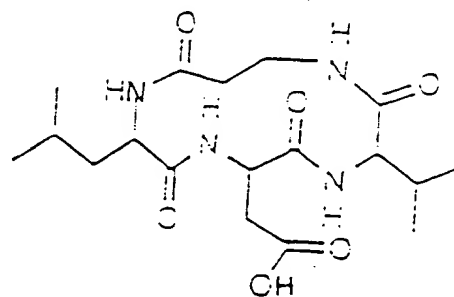
EG-45



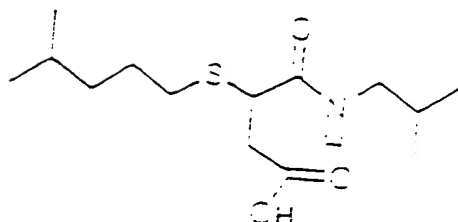
AC-22.



BL-34



NG-93



13-1

SCHEME 1

The compound EG-45 (Scheme 1) is a LDV peptide surrogate that, in addition to lacking the two terminal groups, also has a peptide bond replaced by a peptide bond surrogate. Pseudo-S-CH<sub>2</sub> was chosen since its successful synthesis provides easy access to a new peptide bond replacement for peptides containing an Asp moiety next to the N-terminus. It is expected to be resistant to proteases and its lipophilic character can offer advantages. The synthesis of EG-45 was based on the Michael addition of a thiol to a bis-ester of itaconic acid, by the synthetic path outlined in Scheme 3 herein.

To rule out an irrelevant mechanism of inhibition, compound BL-34 (Scheme 1) was prepared in a way similar to that used for compound EG-45, with sec-butylamine used for the amide formation in the last step of the synthesis, and was used as a control in the cell adhesion studies.

LDV analogues wherein X<sub>2</sub> and X<sub>4</sub> are carbonyl and X<sub>1</sub> and X<sub>3</sub> are oxygen are prepared by Michael addition of malonic acid to diisobutyl maleate followed by decarboxylation. When X<sub>1</sub> and X<sub>3</sub> are nitrogen, Michael addition of malonic acid to maleic acid diisobutyl amide produces the desired compounds.

Pharmaceutically acceptable salts of the LDV surrogates of the invention include but are not limited to inorganic salts, such as sodium, potassium, calcium and the like, and organic salts with amines or organic bases, such as piperidine, morpholine and the like.

The LDV surrogates of the invention can inhibit biological interactions which are dependent on LDV recognition. Examples of versatile recognition processes mediated by the LDV pattern encompassed by the present invention include cellular and molecular interactions involving the LDV sequence by the integrin VLA-4. Thus, they can also prevent metastasis. In addition, the surrogates inhibit lymphocyte interaction with certain antigen-presenting cells and thus inhibit T cell activation and migration, thereby preventing autoimmune diseases.

The compounds of the invention can be administered to

patients by any suitable route including oral and parenteral routes, e.g., intravenous, subcutaneous or intramuscular injection. An effective but essentially non-toxic quantity of the compound is employed in the treatment. Effective amounts may be within the range of 0.01 to 1 mg/kg, preferably 0.5 mg/kg on a regimen in single or more divided daily doses.

The invention further provides a pharmaceutical composition comprising as active ingredient a surrogate according to the invention and a pharmaceutically acceptable carrier. The compositions may be in the form of tablet, capsule, solution or suspension containing from about 0.7 to 70 mg per unit of dosage of an active compound of the invention or mixtures thereof. The compounds may be compounded in conventional manner with a physiologically acceptable vehicle or carrier, excipient, binder, preservative, stabilizer, etc. For example, injections for intravenous administration may be prepared in saline, at a pH level of e.g. 7.4.

The following examples are intended to illustrate, by way of example, the principles of the invention, without limiting it thereto.

#### EXAMPLES

In the following examples, chemical analysis of the synthesized surrogates was carried out by measuring  $^1\text{H}$ - and  $^{13}\text{C}$ -NMR spectra on a Bruker AMX 400 spectrometer operating at 100.75 MHz. Chemical shifts were measured relative to TMS as an external standard.

All starting materials were purchased from Aldrich except for Boc-amino acids which were purchased from Sigma and were used without further purification.

#### Example 1. Preparation of 2,11-dimethyl-6,9-diaza-5,8-dioxo-7-carboxymethyl-dodecane (Compound AC-16)

Compound AC-16 was prepared by coupling with DCC according to the synthesis outlined in Scheme 2.

N,N'-Dicyclohexylcarbodiimide (DCC) (226 mg, 1.1 mmol) was added to a solution containing t-butyloxycarbonyl Asp (O-benzyl) (309 mg, 1.0 mmol) and 1-hydroxybenzotriazole (148

mg, 1.1 mmol) in  $\text{CH}_2\text{Cl}_2$  / THF 1:1 (v/v) solution (7 ml). The reaction was left overnight at room temperature. The solution was filtered to remove dicyclohexylurea (DCU) generated in the reaction, and washed with dry  $\text{CH}_2\text{Cl}_2$ . Isobutyl amine (87 mg, 1.2 mmol) was added and the solution was stirred for 5 h at room temperature. The solution was poured into aqueous 0.1 HCl solution (10 ml) and the product was extracted twice with chloroform (50 ml). The chloroform was dried over  $\text{Na}_2\text{SO}_4$  and was removed under reduced pressure. The crude coupling product was dissolved in  $\text{CH}_2\text{Cl}_2$  (4 ml) and was cooled to  $0^\circ\text{C}$ , trifluoroacetic acid (4 ml) was added and the reaction mixture was allowed to stand 30 min at  $0^\circ\text{C}$  and 1 h at room temperature. The solvent and the acid were removed under reduced pressure, thus producing N-tert-butyloxycarbonyl-4-benzyloxy-L-aspartic acid isobutylamide. The crude product was pure enough for the next step.

N,N'-Dicyclohexylcarbodiimide (DCC) (226, mg, 1.1 mmol) was added to a solution containing isocaproic acid (135 mg, 1.0 mmol) and 1-hydroxybenzotriazole (148 mg, 1.1 mmol) in  $\text{CH}_2\text{Cl}_2$  / THF 1:1 (v/v) solution (7 ml). The reaction was left overnight at room temperature. Dicyclohexylurea (DCU) was filtered off and washed with dry  $\text{CH}_2\text{Cl}_2$ . The crude product from the previous step was added to that solution along with diisopropyl ethyl amine (260 mg, 2 mmol). The reaction mixture was stirred for 5 h at room temperature. The solution was poured into aqueous 5%  $\text{NaHCO}_3$  solution (10 ml) and the product was extracted twice with chloroform (50 ml). The chloroform was dried over  $\text{Na}_2\text{SO}_4$  and was removed under reduced pressure. The product was purified by flash chromatography (ethylacetate : hexane 1:3 ratio) and the benzyl protecting group was removed by hydrogenation (ethylacetate, 10% Pd on charcoal,  $\text{H}_2$ ) at atmospheric pressure. The title compound AC-16 was recrystallized from ethanol.

NMR( $\text{CDCl}_3$ ): 7.08 (1H, d, J=7), 7.01 (1H, d, J=3), 4.75 (1H, ddd, 7, 4, 3), 3.00 (2H, t, J=6), 2.80 (1H, dd, J=16, 4), 2.64 (1H, dd, 16, 7), 2.18 (2H, t, J=7), 1.7 (1H, m), 1.51 (1H, m), 1.49

(2H,m), 0.83 (6H, d, J=6), 0.82 (6H,d, J=7).

FAB MS :  $[M+H]^+ = 287.3$ ,  $[M-H]^+ = 285.3$ .

Example 2. Preparation of 2,11-dimethyl-6,9-diaza-5,8-dioxo-7-carboxyethyl-dodecane (Compound AC-22)

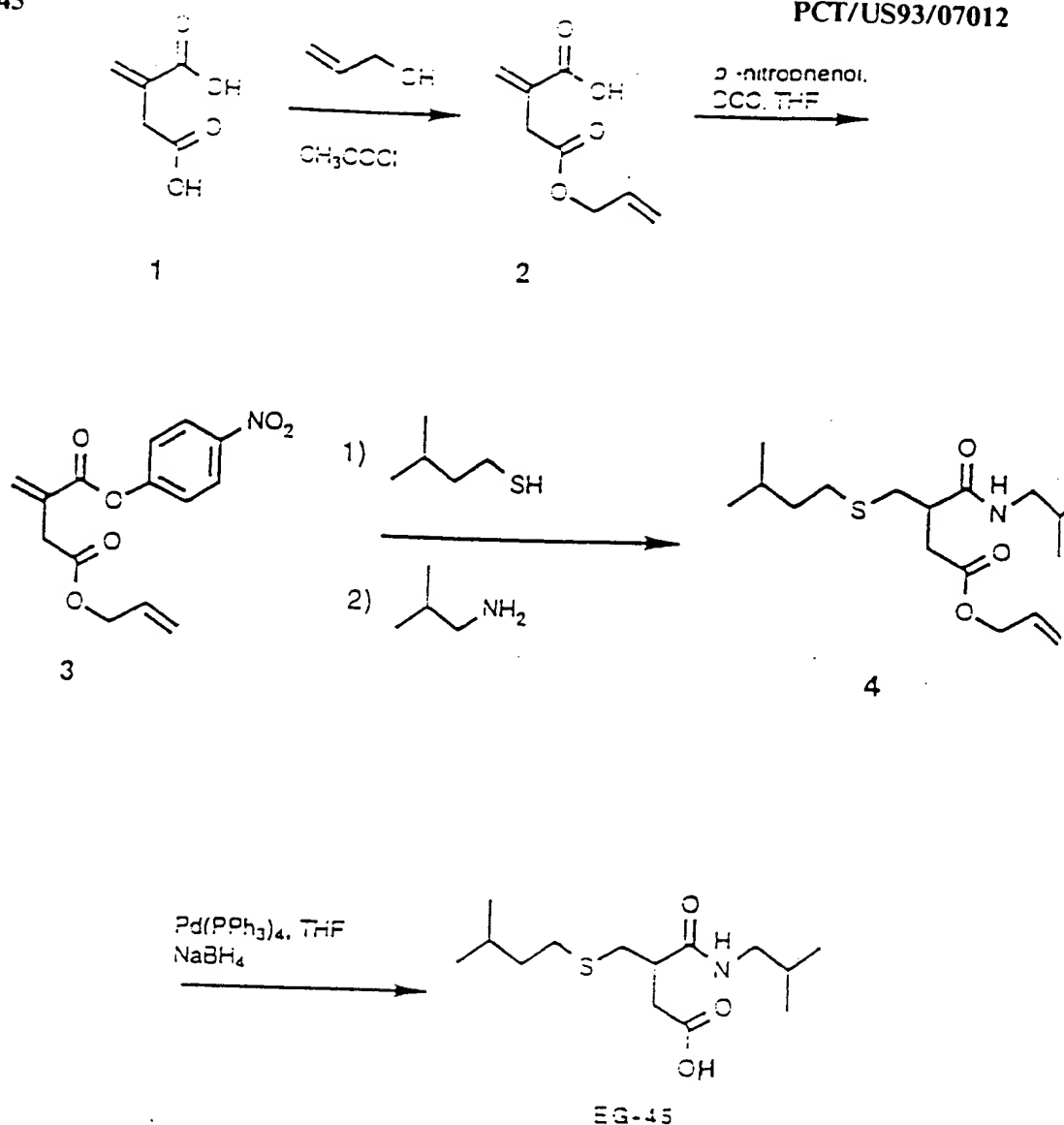
The LEV surrogate AC-22 was prepared by the same procedure as compound AC-16, but using t-butyloxycarbonyl Glu (O-benzyl) instead of the Asp derivative, and was used for comparison with the LDV surrogate compound AC-16.

NMR ( $CDCl_3$ ): 7.39 (1H,t, J=5.6), 6.98 (1H,d, J=8.5), 4.72 (1H,ddd, J=11.0, 8.5, 6), 3.08 (2H,m), 2.48 (1H,ddd, J=16.5, 8.6, 5.3), 2.33 (1H,ddd, J=16.5, 7.2, 5.0), 2.23 (2H, t, J=7.6), 2.06 (1H,m), 1.94 (1H,m), 1.78 (1H,hep, J =6.7), 1.55 (1H,m), 1.51 (2H,m), 0.89 (6H,d, J=6.7), 0.86 (6H,d, J=6.8).

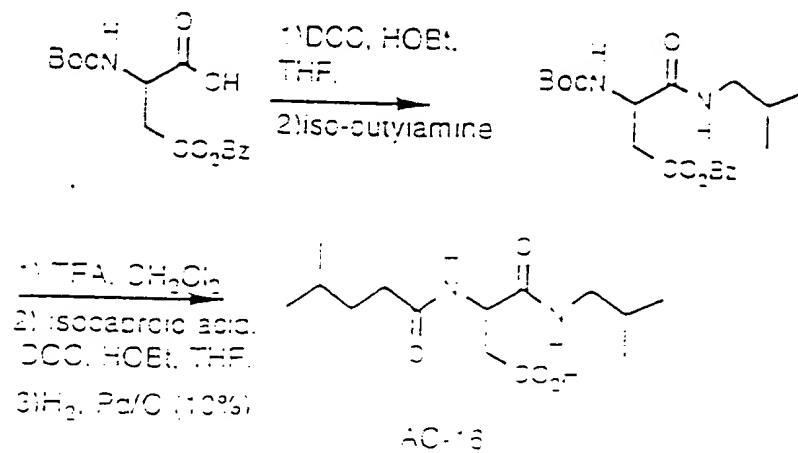
FAB MS :  $[M+H]^+ = 301.3$ ,  $[M-H]^+ = 299.3$ .

Example 3. Preparation of 2,11-dimethyl-6-thia-9-aza-8-oxo-7-carboxymethyl-dodecane (Compound LB-1)

4-methyl-1-pentanethiol (120 mg, 1mM) was added to a solution containing benzyl maleate (206 mg, 1mM) in THF (5 ml). The solution was stirred at room temperature for 12 hours after which the solvent was removed under reduced pressure. The crude mixture was purified by flash chromatography (ethyl acetate : hexane 1:1 as eluent). The pure 1-benzyl-2-[4-methyl-pentylthio] succinate was dissolved in methylene chloride (3 ml) and 1-hydroxybenzotriazole (148 mg, 1.1 mmol) was added followed by DCC (226 mg, 1.1 mmol). The solution was stirred at room temperature for 5 h, the precipitated DCU was filtered off and isobutyl amine (87 mg, 1.2 mmol) was added. The solution was stirred for 5 h at room temperature, poured into aqueous 0.1 HCl solution (10 ml) and the product was extracted twice with chloroform (50 ml). The chloroform was dried over  $Na_2SO_4$  and was removed under reduced pressure. The crude product was purified by flash chromatography using ethylacetate : hexane 1:4 as eluent. The benzyl group was removed by hydrogenation (Pd/C 10% in ethyl acetate,  $H_2$  1 atm) and the final product was recrystallized from ethanol-water mixture.



SCHEME 3



SCHEME 1

Example 4. Preparation of 8-methyl-3-(2-methylpropyl- amino-carbonyl)-5-thianonanoic acid (Compound EG-45)

The synthesis of compound EG-45 was carried out as depicted in Scheme 3 herein. Starting from itaconic acid 1, a monoallyl ester at the  $\beta$ -position 2 is formed under acidic conditions. This was verified by  $^{13}\text{C}$ -NMR which indicates that of the two carbonyl signals of itaconic acid at 166.89 and 171.47 ppm, corresponding to the unsaturated and saturated carbonyls, respectively, only the 171.47 ppm resonance was shifted upfield to 169.58 ppm upon esterification. The same shift was observed for the known itaconic acid  $\beta$ -methyl ester, which was prepared for comparison. An allyl protecting group was chosen because it is easily removed under mild, neutral conditions. It was anticipated that removal of a protecting group under harsh conditions in the last step of the synthesis, could be accompanied by a cyclic imide formation. The p-nitrophenyl ester 3 was prepared using DCC as a coupling agent. Michael addition of 4-methylpentane thiol to p-nitrophenyl ester 3 in DMF at room temperature using diisopropylethylamine as a base, followed by addition of isobutylamine without isolation of the Michael adduct gave allyl ester 4, which was purified by chromatography. Deprotection, using  $\text{Pd}(\text{PPh}_3)_4$  and sodium borohydride in THF yielded the desired product compound EG-45.

4a) Synthesis of  $\beta$ -allyl itaconate 2: Acetyl chloride (0.1 ml) was added to a suspension of itaconic acid 1 (5 g, 38.5 mmol) in allyl alcohol (6.8g, 120 mmol) and the solution was refluxed for 30 min. Excess alcohol was removed under reduced pressure and the resulting oil was dissolved in ether and extracted into 5%  $\text{NaHCO}_3$  solution. The aqueous layer was then acidified and extracted four times with ether. The ether layer was dried with  $\text{Na}_2\text{SO}_4$  and the ether was removed under reduced pressure. The resulting oil (2) was pure enough for the next step.

4b) Preparation of compound 4 : N,N' - dicyclohexylcarbodiimide (DCC) (226 mg, 1.1 mmol) was added to a solution containing  $\beta$ -allyl itaconate 2 (170mg, 1.0 mmol) and p-

nitrophenol (153 mg, 1.1 mmol) in  $\text{CH}_2\text{Cl}_2/\text{THF}$  1:1 (v/v) solution (7 ml). The reaction was left for 18-hr at 22°C, filtered to remove DCU, and washed with dry  $\text{CH}_2\text{Cl}_2$ . Isoamyl thiol (208 mg, 1mmol) was added to the crude p-nitrophenyl ester 3 and diisopropylethylamine (260 mg, 2 mmol) was added. The solution was stirred at 22°C until no starting material could be detected by TLC (developed with ethylacetate:hexane 1:4 and sprayed with phosphomolibdic acid). Isobutyl amine (70 mg, 1mmol) was added and the solution was stirred for another 5 hr. The solvent was then removed under reduced pressure and the crude reaction mixture was flash chromatographed (ethyl acetate/hexane; 1:8) to yield pure compound 4 (200 mg, 50% yield).

4c) Preparation of compound EG-45: In order to remove the protecting groups, tetrakis (triphenylphosphine) palladium (57 mg, 0.05 mmol, 10 mol%) was added to a solution of pure 4 (200 mg, 0.5 mmol) and sodium borohydride (40 mg, 1 mmol) in dry tetrahydrofuran. The solution was left stirring under an argon atmosphere for 3 hr, after which no starting material could be detected by TLC. The solution was poured into 5% aqueous  $\text{NaHCO}_3$  solution and washed with ether. The aqueous layer was then acidified with dilute HCl and extracted with ether. The resulting product EG-45 was judged pure by  $^1\text{H-NMR}$  and TLC (one spot, ethyl acetate as eluant).

NMR( $\text{CDCl}_3$ ): 6.15 (1H, br t,  $J=6.6$ ), 3.08 (2H, t,  $J=6.6$ ), 2.82-2.59 (5H, m), 2.50 (2H, t,  $J=7.7$ ), 1.76 (1H, 7 lines,  $J=6.6$ ), 1.60 (1H, 7 lines,  $J=6.7$ ), 1.41 (2H, q,  $J=7.7$ ), 0.89 (6H, d,  $J=6.7$ ), 0.87 (6H, d,  $J=6.6$ ).

FAB MS:  $[\text{M}+\text{H}]^+=290.2$ ,  $[\text{M}-\text{H}]^-=288.2$ .

Analysis: calculated for  $\text{C}_{14}\text{H}_{27}\text{NO}_3\text{S}$ : C, 58.13, H 9.34, N 4.84, S 11.06. Found: C, 57.85, H 9.06, N 4.74, S 10.66.

Example 5. Preparation of 8-methyl-3-(1-methylpropylamino-carbonyl)-5-thianonanoic acid (Compound BL-34)

Compound BL-34, used for comparison with EG-45, was prepared by the same procedure as compound EG-45 in Example 4, but using sec-butylamine instead of the isobutylamine. The



resulting mixture of diastereomers was used without further separation.

NMR(CDCl<sub>3</sub>): 5.97(1H,d,J=8.4), 3.89(1H,m), 2.80(2H,m), 2.79-2.59(3H,m), 2.53(2H,t,J=7.7), 1.46(1H,m), 1.13(1H,q, J=6.6), 0.93(6H,m), 0.89(6H,d,J=6.6).

FAB MS: [M+H]<sup>+</sup>=290.1, [M-H]<sup>-</sup>=288.3

#### Example 6. Preparation of Compound NG-93

N- $\alpha$ -tBoc-Valine was coupled to an oxime resin (DeGrado, W.F. and Kaiser, E.T., 1980, J. Org. Chem. 45:1295) using DCC as the coupling reagent. Thus oxime resin (2.00g) was added to a solution containing N- $\alpha$ -tBoc-valine (1 mmol) and DCC (1 mmol) in methylene chloride (10 ml). The mixture was shaken for 15 h, washed with methylene chloride, DMF, isopropanol and methylene chloride and dried in vacuo. The rest of the synthesis was carried out manually coupling being carried out with 3 equivalents of the appropriate tBoc-amino acid symmetric anhydrides; (Asp and then Leu). The peptide was cleaved from the resin with a 3 fold excess of  $\beta$ -alanine-O-t-butyl acetate in DMF/CH<sub>2</sub>Cl<sub>2</sub> 1:1 for 4h and was washed from the resin with DMF. Deprotection of the t-butyl ester and t-butyloxycarbonyl groups was achieved by treatment in TFA:CH<sub>2</sub>Cl<sub>2</sub> 1:1 for 30 min and 1h at room temperature. The solvent and the acid were removed under reduced pressure and cyclization was achieved with DCC and 1-hydroxybenzotriazole in CH<sub>2</sub>Cl<sub>2</sub>. Hydrogenolysis of the benzyl protecting group was carried out with ethylacetate, 10% Pd on charcoal and H<sub>2</sub> at atmospheric pressure. The final product was purified by reverse phase chromatography followed by preparative HPLC purification.

All compounds prepared by this method had NMR and FAB-MS spectra consistent with the proposed structures.

#### Example 7. Inhibition of T cell adhesion to ECM proteins

Cell recognition via attachment to the ECM and its FN-glycoprotein component is a complex process involving multiple cell-ligand interactions. The high frequency of the V segment in ECM-derived FN, as compared to that found in

plasma FN implies that the VLA-4 integrin plays a major role in cellular interactions with immobilized FN. Indeed, this integrin was shown to participate in various processes, such as lympho-hemopoiesis, bone marrow cell differentiation, VCAM-1 recognition, cell activation, and the binding of FN to several cell types, including tumor cells and lymphocytes (Elices et al, 1990; Shimizu et al, 1990; Roldan et al, 1992). The minimal recognition site on FN for the VLA-4 integrin appears to be restricted to the alternatively spliced V region and is blocked specifically by the LDV-containing peptides (Humphries et al, 1986).

#### 7a. Preparation of CD4<sup>+</sup>T cell.

CD4<sup>+</sup>T cells were purified from the peripheral blood of healthy human donors. Mononuclear leukocytes were isolated using Ficoll gradients, washed and incubated in RPMI supplemented with 10% FCS and antibiotics in petri dishes at 37°C humidified CO<sub>2</sub> incubator. After 2 h, the non-adherent cells were isolated and applied on nylon-wool columns (1.5 h). The CD4<sup>+</sup> T cells were then negatively selected by exposure of these cells to a mixture of anti-CD8, CD19, and CD14 monoclonal antibodies (mAb) conjugated to magnetic-beads (Advanced Magnetix, MA). Cells that did not bind to the beads were exposed to a second round of negative selection. The resulting cell population, which consisted of over 90% CD3<sup>+</sup> CD4<sup>+</sup> cells, as determined by FACScan analysis (not shown), was used as the source of human CD4<sup>+</sup>T cells.

#### 7b. T cell adhesion to ECM proteins.

To examine the adhesive properties of human CD4<sup>+</sup>T cells to ECM components, ECM components were first bound to polystyrene wells. FN or laminin (LN) (Sigma; 1 mg/50 ml medium/well) was added to 96-well flat-bottom microtiter plates (Costar) and incubated for 2 hours. Unbound protein was removed by washing and the remaining binding sites on the plates were blocked by 1% BSA (Sigma) in PBS. The T cells were radioactively labeled with <sup>51</sup>[Cr] (New England Nuclear) and activated with phorbol myristate acetate (PMA;

Sigma; 10ng/ml). Where indicated, these T cells were pretreated (30 min at 37°C) with anti-CD29 (anti- $\beta_1$  chain of VLA integrins; diluted 1/200, Serotec, Oxford, UK), anti-VLA-5, anti-VLA-4, or anti-VLA-6 (diluted 1/400; Telios Pharmaceuticals Research Inc.) mAb. The RGD peptide GRGDSPK was purchased from Sigma. The LDV peptide EILDVPST was synthesized by an Applied Biosystems synthesizer at the Peptide Synthesis Unit, The Weizmann Institute of Science, Rehovot, Israel. The  $^{51}\text{Cr}$ -labeled CD4<sup>+</sup>T cells ( $2 \times 10^5$ /well) were then added to the coated microtiter plates and incubated for 30 min at 37°C in a 10% CO<sub>2</sub> humidified incubator. The non-adherent cells were then gently removed and the adherent cells were lysed and collected. The percent of cells that were adhered was calculated as follows: [CPM of residual cells in the well / (total CPM of cells added to the well - spontaneous release of  $^{51}\text{Cr}$ )  $\times$  100]. Results are expressed as the mean percent of T cell binding derived from quadruplicate wells for each experimental group.

7c. Inhibition of T cell adhesion to ECM proteins by LDV and RGD peptides

To verify and demonstrate that T-cell adhesion to FN and LN is indeed mediated by the  $\beta_1$ -subfamily of integrins, the receptor requirements for human CD4<sup>+</sup>T cell-adhesiveness to FN were studied. Purified human CD4<sup>+</sup>T cells were radioactively labeled with  $^{51}\text{Cr}$ , treated with various molecules or mAbs, and then seeded in wells coated with FN. LN, a major component of the ECM, served as a control ECM-adhesive glycoprotein. The T lymphocytes were then activated with PMA, and following incubation, the percent of labeled CD4<sup>+</sup>T cells attached to the protein substrate was measured. The results shown in Table 1 indicate that T cells adhere to the major adhesive ECM glycoproteins, FN and LN, primarily via their  $\beta_1$ -integrin subfamily of receptors. Cell adhesion to FN was verified to depend on both VLA-4 ( $\alpha_4\beta_1$ ), and VLA-5 ( $\alpha_5\beta_1$ ) integrins, whereas T cell adhesion to LN is primarily mediated via VLA-6 ( $\alpha_6\beta_1$ ). As expected, anti-CD29 mAb specific to the common  $\beta_1$ -chain of VLA

integrins, abolished T cell adhesion to both adhesive glycoproteins.

To analyze the specific inhibition of T cell adhesion to FN by LDV and RGD peptides, the inhibitory capacities of these peptides at a fixed concentration of 600  $\mu\text{g/ml}$  were analyzed. Both reagents appeared to inhibit the CD4<sup>+</sup>T cell-FN adhesive interaction, although the RGD-peptide inhibition was stronger than that of the LDV-containing peptide (80 and 46% inhibition, respectively). This mode of inhibition was specific: neither peptide interfered with the integrin-mediated interactions of the T cells with LN (Table 1).

Table 1. Involvement of  $\beta_1$ -integrins in the adhesion of human CD4<sup>+</sup> T cells to FN and LN.

Inhibitor of cell adhesion	Percent T cell adhesion to:	
	FN	LN
None	60 $\pm$ 6	50 $\pm$ 5
mAb to:		
CD-29 ( $\beta_1$ )	11 $\pm$ 3	10 $\pm$ 4
VLA-4 ( $\alpha_4$ )	25 $\pm$ 3	47 $\pm$ 5
VLA-5 ( $\alpha_5$ )	18 $\pm$ 4	53 $\pm$ 3
VLA-6 ( $\alpha_6$ )	57 $\pm$ 4	14 $\pm$ 3
Peptides		
EELDVPSY	34 $\pm$ 3	46 $\pm$ 4
GRCDSP	13 $\pm$ 2	48 $\pm$ 3

7d. Inhibition of T cell adhesion to FN by the LDV surrogates

In view of the ability of the LDV peptide to interfere with T cell-FN adhesiveness, we examined the ability of the LDV surrogates of the invention to competitively inhibit CD4<sup>+</sup> human T lymphocyte binding to the ECM-ligand.

The inhibitory effect of the LDV surrogates on cell adhesion to FN was measured in a way similar to that described for peptide inhibition. The freshly isolated and purified human CD4<sup>+</sup>T cells were pretreated with the inhibitory compounds (peptides GRGDSP and EILDVPST, the LDV surrogates AC-16 and EG-45, and comparison compounds AC-22 and BL-34) at a fixed concentration of 600 µg/ml. The cells were then seeded in the FN or LN precoated wells. Percent cell adhesion was calculated thereafter. Values ±SD are shown in Fig. 1 (one experiment representative of 3).

The results shown in Fig. 1 indicate that surrogates AC-16 and EG-45 inhibited T cell adhesion to FN. The AC-16 mimetic is almost as active as the LDV-containing peptide, while AC-22 is inactive, indicating that the amino and carboxyl terminal groups of the tripeptide LDV are not required for its activity. EG-45 was a better inhibitor of T cell adhesion than both AC-16 and the LDV-containing peptide (54, 34, and 46%, respectively). Compounds AC-22 and BL-34 did not inhibit T cell adhesion to neither ECM-glycoproteins. AC-22, derived from glutamic acid, can be regarded as an LEV surrogate, and its inability to inhibit cell adhesion reflects the situation observed with the LEV-NH<sub>2</sub> peptide (Humphries et al, 1986). In compound BL-34, a terminal methyl group from the "amide side" was "shifted" to a position closer to the nitrogen, presumably introducing steric hindrance and interfering with hydrophobic group binding to its receptor pocket. This probably accounts for the poor inhibitory potential of this compound on CD4<sup>+</sup>T cell adhesion to FN.

That none of the surrogates interfered with T cell adhesion to LN indicates that the inhibitory effect of the LDV analogs cannot be attributed to a toxic effect exerted by

these molecules. Analogously, none of the indicated surrogates inhibited a lectin (PHA)-induced T-cell proliferative responses and TNF- $\alpha$  secretion (data not shown).

To compare the kinetics of the inhibitory effect of the LDV analogues on CD4<sup>+</sup> T cell adhesion to FN, the cells were treated with the various inhibitors at 200, 400 and 800  $\mu\text{g/ml}$ , and their ensuing FN-adhesiveness was examined. Values  $\pm$ SD are shown in Fig.2 (one experiment representative of 4). The results, shown in Fig.2, indicate that (a) compounds AC-16 and BL-34 had no effect; (b) at 400 and 800  $\mu\text{g/ml}$ , compound EG-45 was a better inhibitor of cell adhesion than AC-16 and the LDV-containing peptide; and (c) an amount of 200  $\mu\text{g/ml}$  of the particular inhibitors was only marginally effective in inhibiting cell adhesion to immobilized FN. Note that at a concentration of 800  $\mu\text{g/ml}$ , both the LDV peptide and the AC-16 surrogate exerted a similar inhibitory effect.

The inhibitory capacities of the LDV-peptide and EG-45 surrogate on cell attachment to FN was compared at various concentrations. The results indicate that while as the effective dose (ED)<sub>50</sub> of the two compounds were quite similar, i.e. 275 and 310  $\mu\text{g/ml}$ , respectively (Fig.3), the maximal inhibitory effect of compound EG-45 was markedly higher than that of the LDV peptide (53 vs 36% at 1  $\mu\text{g/ml}$ , respectively). Thus, compound EG-45 could be considered as a potent antagonist of integrin recognition of FN and the subsequent adhesive interactions, indicating that the LDV mimetics can inhibit T cell mediated immune responses also in-vivo. It had been shown that treatment of mice with a synthetic EILDVPST peptide inhibited the induction of contact sensitivity, a T cell mediated immune reaction in the dermal micro-environment of mice (Ferguson et al, 1991). Our results strongly suggest that the LDV surrogates may indeed serve as inhibitors of inflammatory reactions.

It appears from these experiments that the integrin binding site of the LDV sequence is composed of two hydrophobic pockets that surround a binding site for the Asp-carboxylate group. Interestingly, the Asp residue has been

implicated to be involved in many integrin-binding ligands. Our results suggest that the carboxyl and amino groups at the C and N terminals of the peptide make only a minor contribution to the inhibitory effect of LDV containing peptides. Accordingly, a replacement of at least one peptide bond does not reduce the affinity for the receptor. This means that the peptide backbone as well makes no direct contribution to the affinity of the integrin for the LDV ligand. However, the backbone may serve to position the hydrophobic and hydrophilic groups in the appropriate orientation for integrin-recognition and binding. Based on the findings described herein (and that of our previous study on the analysis RGD surrogates), we may conclude that a relatively flexible compound can acquire substantial binding affinity to integrin receptors. It is reasonable to assume, however, that a more conformationally constrained mimetics of the LDV cell adhesion motif might acquire considerably higher binding affinities to its corresponding functional site on integrin(s).

The results of these experiments and our previous in-vitro and in-vivo investigation of RGD-nonpeptidic mimetics (PCT/US92/09951) indicate that the compounds of the invention may specifically interfere with integrin-mediated cell recognition of FN, and thus provide a new approach to therapeutic tools, for example, in pathological disorders in which the recognition of blood-vessel-wall components by extravasating cells is required.

**Example 8. Inhibition of DTH response in vivo to OX by treatment of mice with LDV surrogates**

The interference of LDV surrogates with T cell-mediated immune response in vivo was tested. A delayed-type hypersensitivity (DTH) reaction experiment was performed in which groups of BALB/c mice (5 mice per group) were sensitized on the shaved abdomen with the skin allergen 4-ethoxymethylene-2-phenyl-oxazolone (OX) (10  $\mu$ l of 3% OX in acetone/olive oil) and challenged 5 days later by applying OX to their ears. The increment in ear swelling was recorded 24h

later as a measure of DTH. The mice were treated following their OX-priming with intravenous injections of 25  $\mu$ g of the LDV-containing peptide or the LDV-mimetic AC-16, or with PBS. The results are shown in Table 2.

The purpose of this experiment was to examine the possible usage of the LDV-analogue on T-cell mediated immune response in vivo. The results shown here strongly indicate that AC-16 can indeed serve as a powerful inhibitor of T-cell immunity, being this capable of inhibiting autoimmune disorders, allergy and other processes which involve T-cell migration through ECM.

TABLE 2  
Specific Inhibition of DTH Response in vivo to OX  
by Treatment of Mice with Compound AC-16

Treatment of Mice	Measurement of OX-mediated DTH response	
Compound	Ear swelling ( $\times 10^{-2}$ mm $\pm$ SD)	% Inhibition
None	21 $\pm$ 2	-
LDV	18 $\pm$ 3	14
PBS	22 $\pm$ 3	0
AC-16	13 $\pm$ 2	39

The results shown here indicate that daily administration of the LDV-mimetic, but not the LDV-peptide or PBS, inhibited markedly DTH response in vivo.

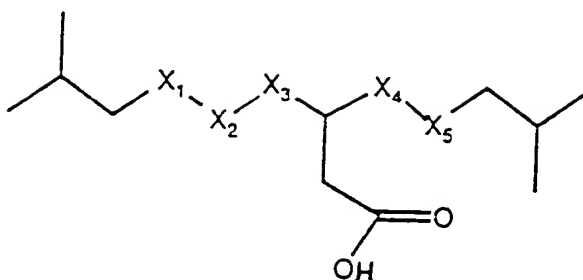


REFERENCES

- Elices, M.J. et al. (1990) Cell, 60:577-584.
- Ferguson, T.A. et al. (1991) Proc Natl Acad Sci U.S.A., 88:8072-8076.
- Guan, J.L. and R.O. Hynes (1990) Cell, 60:53-61.
- Humphries, M.J. et al. (1986) J. Cell Biology, 103:2637-2647.
- Nojima, Y. et al. (1990) J. Exp. Med., 172:1185-1192.
- Postigo, A. et al. (1991) Eur J Immunol, 21:2437-2445.
- Roldan, E. et al. (1992) J. Exp. Med., 175:1739-1747.
- Shimizu, Y. et al. (1990) J. Immunol., 145:59-67.

CLAIMS

1. A compound of the general formula I

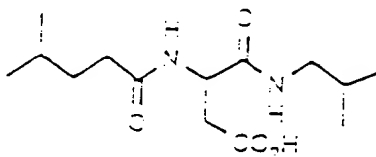


wherein

X<sub>1</sub> to X<sub>5</sub> are the same or different C, N, O or S atoms, at least two of them being C, and the X<sub>1</sub>-X<sub>5</sub> chain may be optionally substituted by radicals selected from halogen, hydrocarbyl, oxo, thioxo, amino and carboxyl, or the X<sub>1</sub>-X<sub>5</sub> chain or part thereof may form part of a heterocyclic ring, and pharmaceutically acceptable salts thereof.

2. A compound as claimed in claim 1 wherein X<sub>1</sub>, X<sub>2</sub> and X<sub>4</sub> are C and X<sub>3</sub> and X<sub>5</sub> are N.

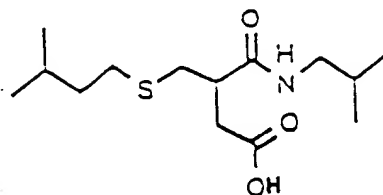
3. A compound as claimed in claim 2 of the formula



AC-16

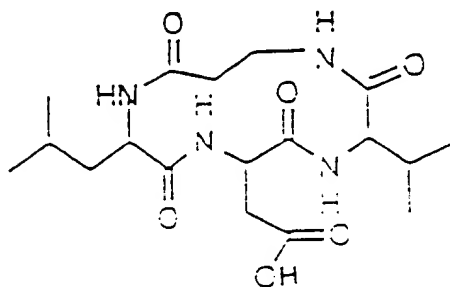
4. A compound as claimed in claim 1 wherein  $X_1$ ,  $X_3$  and  $X_4$  are C,  $X_2$  is S and  $X_5$  is N.

5. A compound as claimed in claim 4 of the formula



EG-45

6. A compound as claimed in claim 1 wherein  $X_1$ ,  $X_3$  and  $X_4$  are C,  $X_2$  and  $X_5$  are N and together with the C adjacent to  $X_5$  they form a 13-membered heterocyclic ring, of the formula



NG-93

7. A compound as claimed in any of claims 1 to 6 which inhibit cellular or molecular interactions which depend on recognition of the LDV sequence.

8. A pharmaceutical composition comprising a compound of formula I in claim 1, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

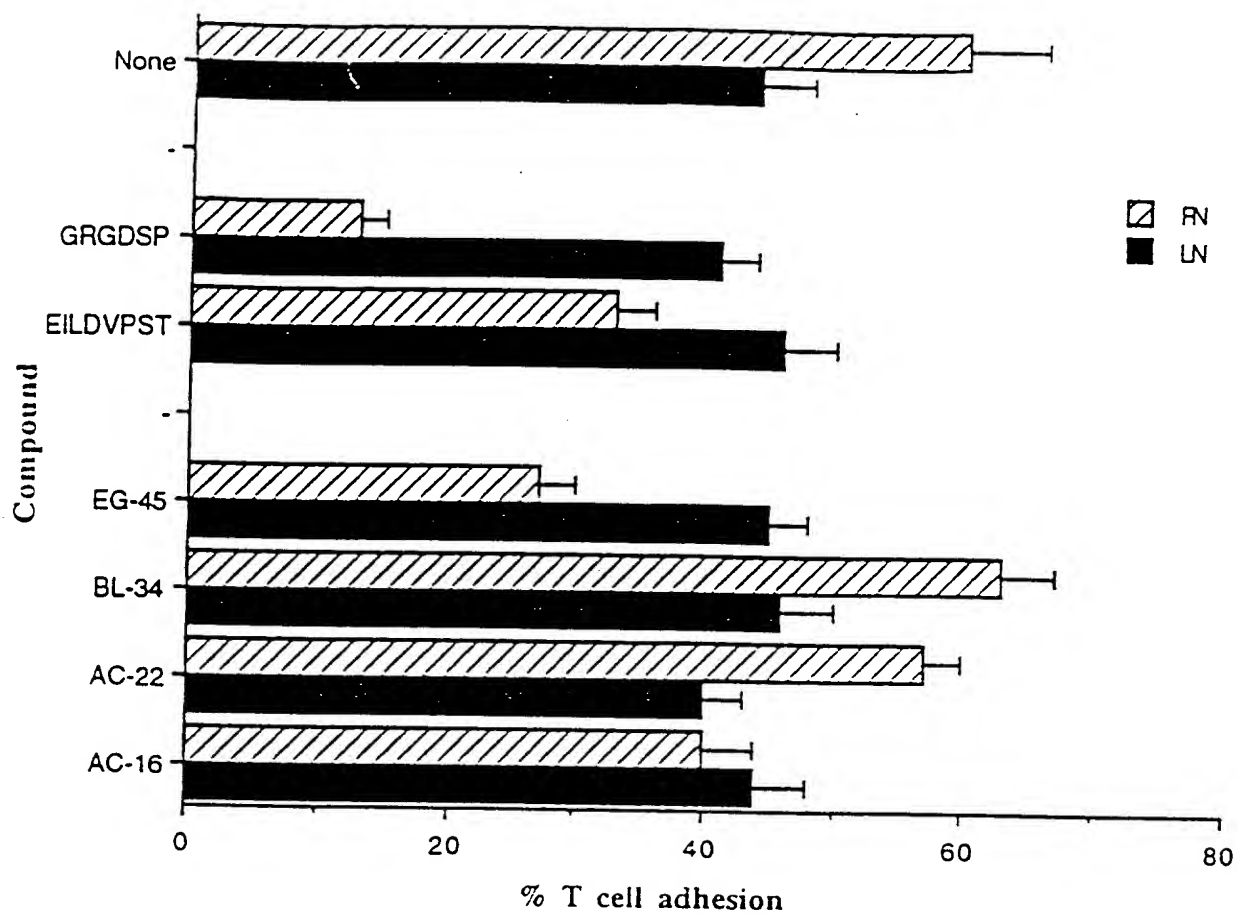


Fig. 1

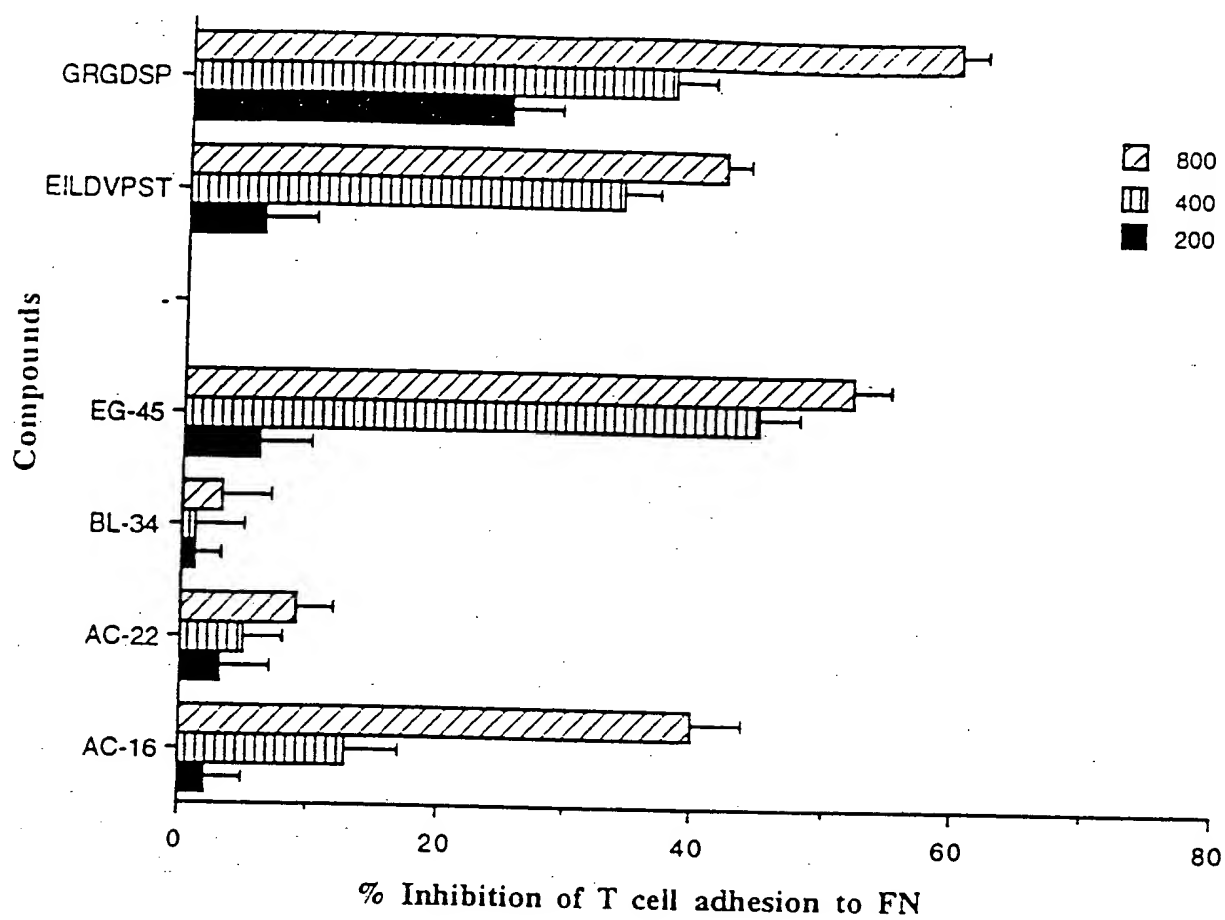


Fig. 2

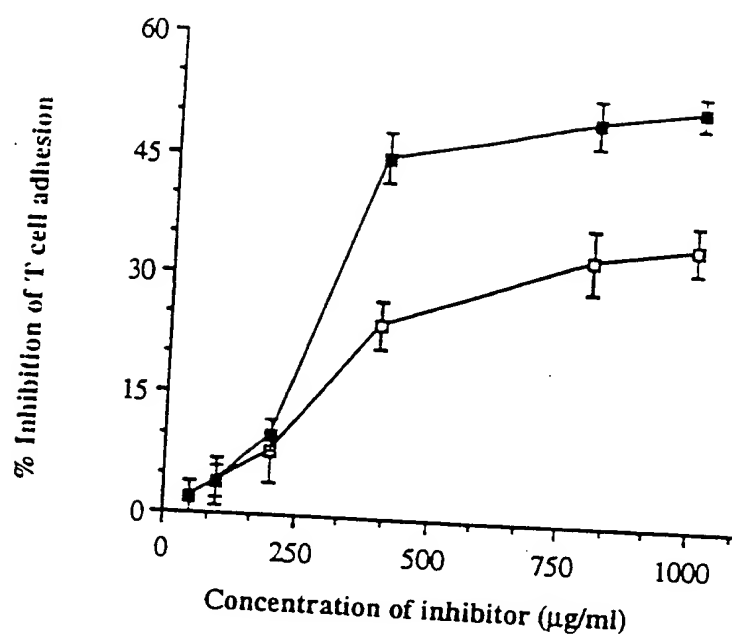


Fig. 3

## INTERNATIONAL SEARCH REPORT

International Application No

PC1/US 93/07012

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07C237/06 A61K31/16 C07C323/60 C07K7/56

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07C C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	JOURNAL OF BIOLOGICAL CHEMISTRY vol. 266, no. 23, 15 August 1991, BALTIMORE, MD US pages 15075 - 15079 Komoriya A et al 'The minimal essential sequence for a major cell type-specific adhesion site (CS1) within the alternatively spliced type III connecting segment domain of fibronectin is leucine-aspartic acid-valine' see page 15077, right column, line 3 - page 15078, right column, line 44 -----	1,7
A	WO,A,91 03252 (WAYNER, ELIZABETH) 21 March 1991 see abstract; page 29, line 31 - page 30, line 32; claims 1, 6-10, 25-29 -----	1,7,8

☐ Further documents are listed in the continuation of box C.☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

25 October 1993

Date of mailing of the international search report

- 9. 11. 93

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SECRET

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCI/US 93/07012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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WO-A-9103252

21-03-91

AU-A-

6354290

08-04-91

CA-A-

2065292

02-03-91

EP-A-

0489837

17-06-92

JP-T-

5503070

27-05-93